

# SPECIFICATION

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## [ ***METHOD FOR RAISING THE BLOOD GLUCOSE LEVEL IN MAMMALS*** ]

### Cross Reference to Related Applications

#### Referenced-applications

This application is a divisional application of co-pending USSN 09/365,404 filed August 2, 1999.

### Background of Invention

[0001] The invention relates to a method in which, by <sup>inhibitory!</sup> reducing dipeptidyl peptidase IV (DP IV) or DP IV-analogous enzyme activity in the blood of a mammal by administration of activity-reducing effectors, the endogenous (or additionally exogenously adminisglycogenolytically active peptide glucagon or analogues thereof is/are degraded to a reduced extent by DP IV and D IV-like Penzymes, thereby reducing or delaying the decrease in concentration of that peptide hormone or analogues thereof.

[0002] Owing to that increased stability of (endogenous or exogenously administered) glucagon and its analogues brought about by the action of DP IV effectors, thereby making them available in greater number for the glycogenolytic stimulation of the glucareceptors of, in particular, liver cells, the duration of activity of the body's own glucagon changes, consequently resulting in stimulation of the catabolic carbohydrate metabolism of the organism treated.

[0003] As a result, the blood sugar level in the serum of the treated organism rises above the glucose concharacteristic of hypoglycaemia. Thereby, metabolic anomalies, such as hypostates resulting from reduced glucose concentrations in the blood, can be

prevented or alleviated.

[0004] In addition to proteases involved in non-specific proteolysis, which ultimately causes the degradation of proteins into amino acids, regulatory proteases are known which take part in the functionalisation (activation, deactivation, modulation) of endogenous peptide active substances (Kirschke *et al.*, 1995; Kr ä usslich & Wimmer, 1987). Especially in connection with immune research and neuroresearch, a number of such so-called convertases, signal peptidases or enkephalinases have been discovered (Gomez *et al.*, 1988; Ansorge *et al.*, 1991).

[0005] In view of the frequency with which the amino acid proline occurs in a large number of peptide hormones and the associated structural characteristics of those peptides, a function analogous to that of the signal peptidases is being discussed for proline-specific peptidases (Yaron & Naider, 1993; Walter *et al.*, 1980; Vanhoof *et al.*, 1995). By its special structure, proline in those peptides determines both conformation and stability of those peptides by protecting them from being degraded by non-specific proteases (Kessler, 1982).

[0006] Enzymes that have a highly specific structure-altering effect on proline-containing sequences (HIV protease, cyclophilin etc.) are attractive targets for current active substance research. In particular for the peptidases prolyl endopeptidase (PEP) and dipeptidyl peptidase IV (DP IV) which cleave after the proline, relationships between modulation of the bioactivity of natural peptide substrates and their selective cleavage by those enzymes could be made plausible. Thus, it is assumed that PEP plays a role in learning and in the memory process and that DPis involved in signal transmission during the immune response (Ishiura *et al.*, 1990; Hegen *et al.*, 1990).

[0007] As with the extraordinary proline specificity of those enzymes, there is discussion about their high selectivity for the amino acid alanine inside typical recognition regions in substrates of those enzymes, according to which alanine-conpeptides can adopt similar conformations to the structurally analogous proline-containing peptides. Such properties of alanine-containing peptide chains have recently been demonstrated by point mutation (exchange of proline for alanine) (Dodge & Scheraga, 1996).

[0008] DP IV and DP IV-analogous activity (for example cytosolic DP II has a substrate specificity virtually identical to that of DP IV) occurs in the blood circulation where it removes dipeptides from the N-terminus of biologically active peptides in a highly specific manner when proline or alanine form the adjacent residues of the N-terminal amino acid in their sequence. On the basis of that cleavage site specificity, it is assumed that that enzyme and analogues are involved in the regulation of polypeptides *in vivo* (Vanhoof *et al.*, 1995).

[0009] Blood sugar concentrations that are too low may lead to pathological states in the human or animal organism. In particular, after accidents, so-called hypoglycaemic shock may occur which may lead in patients to hyperorexia, sweating and even to loss of consciousness and death.

[0010] It was therefore a problem of the present invention to provide agents for preventing or alleviating pathological metabolic anomalies of mammalian organisms, such as acute or chronic hypoglycaemia.

[0011] In particular, it was a problem of the present invention to provide agents by means of which carbohydrate reserves, for example of the liver, can be rapidly mobilized.

[0012] Those problems are solved according to the invention by the use of activity-reducing effectors of dipeptidyl peptidase IV (DP IV) and DP IV-analogous enzyme activity to raise the blood sugar level in a mammalian organism.

[0013] It is already known to use activity-reducing effectors of DP IV to lower the blood sugar level of mammalian organisms. In so doing, the degradation of incretins, which stimulate glucose degradation, by DP IV is stopped.

[0014] It is therefore especially surprising that activity-reducing effectors of DP IV and DP IV-analogous enzyme activity can be used to raise the blood sugar level. Presumably, that effect relies on the following mechanisms:

[0015] In glucose metabolism and catabolism in the human and animal body, a distinction can be made in principle between two phases:

[0016] 1. In the first phase, following food intake, increased release of incretins takes

place (i.e. hormones that stimulate insulin secretion of the pancreas, such as gastric inhibitory polypeptide 1-42 (GIP<sub>1-42</sub>) and glucagon-like peptide amide-1 7-36 (GLP-1<sub>7-36</sub>)), resulting in increased insulin production and, as a consequence, in increased degradation of the glucose supplied by food intake.

[0017] Incretins are, however, substrates of DP IV, since the later is able to remove the dipeptides tyrosinyl alanine and histidyl alanine from the N-terminal sequences of incretins *in vitro* and *in situ* (Mentlein *et al.*, 1993). Consequently, if DP IV is present, degradation of the incretins occurs, which in turn leads to reduced glucose degradation.

[0018] By inhibiting the DP IV and DP IV-analogous enzyme activity *in vivo*, therefore, it is possible effectively to suppress excessive degradation of the incretins and consequently to increase glucose degradation:

[0019] - DP IV inhibition leads to stabilisation of the incretins,

[0020] - the extended life of the incretins in the blood circulation intensifies their insulinotropic and insulin-sensitising action,

[0021] - the consequently increased and more effective insulin release brings with it an increased glucose tolerance (Demuth *et al.*, 1996).

[0022] It has been demonstrated in diabetic rats that the corresponding DP IV inhibitors can be used effectively to modulate the control system described (Pederson *et al.*, 1998). That phase lasts approximately 120 from the time of food intake.

[0023] After that so-called postprandial phase has elapsed, the secretion of incretins is stopped and any already existing incretins are degraded by DP IV. As a result, insulin production falls, bringing glucose degradation to an end.

[0024] 2. In order to maintain the physiologically necessary glucose concentration of approximately 5.5 mmol/l between food intakes, in the second phase stored glycogen is degraded, for which glucagon is released from the pancreatic A-cells. Glucagon has, therefore, an opposite effect to insulin and hence also to the incretins.

[0025] In the case of three meals a day, the human body is accordingly under GLP-1/GIP

and insulin control for approximately 6 hours (3 x 120 minutes), but under glucagon control for 18 hours.

[0026] It has been established that DP IV is endogenously released from the same secretory granules of the A-cells as glucagon and that that release may take place simultaneously with the release of glucagon and the onset of glucagon action. According to the invention, it has now been found that glucagon both *in vitro* and *in vivo* is degraded and thereby deactivated by DP IV and DP IV-analogous enzyme activity, see Figure 1, as a result of which the release of glycogen and consequently of glucose is retarded or stopped. That fact was completely surprising, since it had previously been assumed that, as mentioned above, DP IV causes only lowering of the blood sugar level.

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## Summary of Invention

- [0039] The possibility therefore presents itself, according to the invention, of promoting the release of endogenous stored glucose from glycogen by means of glucagon by influencing DP IV activity and analogous activities; simultaneous stimulation of glucose degradation does not occur, since no incretins are secreted in the human organism approximately 2 hours after meals. 
- [0040] The invention is based, therefore, on the surprising discovery that a reduction of the DP IV or DP IV-like enzyme activity taking place in the blood circulation leads causally to influencing of the blood sugar level. It has been found that: 

[0041] 1.reduction of DP IV and DP IV-analogous activity results in increased stability of externally supplied or endogenously circulating glucagon, that is to say, by administering effectors of DP IV and DP IV-analogous proteins, glucagon degradation in the blood can be controlled;

[0042] 2.by increasing the stability of endogenously circulating or externally supplied glucagon, a controllable modulation of the blood glucose level occurs.

[0043] The invention accordingly relates to the use of effectors of dipeptidyl peptidase IV (DP IV) and DP IV-analogous enzyme activity to raise the blood sugar level in the serum of a mammalian organism above the glucose concentration characteristic of hypoglycaemia.

[0044] The invention relates especially to the use and administration of effectors of DP IV and DP IV-analogous enzyme activity in and to mammals for the prevention or alleviation of pathological metabolic anomalies of mammalian organisms. Such an anomaly may be, for example, acute or chronic hypoglycaemia where rapid mobilization of carbohydrate reserves of the liver is necessary.

[0045] In another preferred embodiment, the invention relates to use and a method for raising the blood sugar level in the serum of a mammalian organism above the gluconcentration characteristic of hypoglycaemia. For that purpose, a therapeutically effective amount of an effector of DP IV and DP IV-analogous enzyme activity may be administered to a mammalian organism.

[0046] A significant advantage of the present invention is the low burden imposed on the organism, since only small doses of external hormone, if any, need to be administered: according to the invention, glucagon degradation is decelerated or completely stopped by the use of the DP OV inhibitors of the invention, so that, in the organism of an adult human, typically a quantity of administered or endogenously released glucagon of from 2 pmol to 200 pmol is maintained. Too rapid a proteolytic degradation is prevented.

[0047] The effectors of DP IV and DP IV-analogous enzymes administered according to the invention may be used in pharmaceutically administrable formulation complexes as inhibitors, substrates, pseudosubstrates, inhibitors of DP IV expression, binding

proteins or antibodies to those enzyme proteins or combinations of those different substances that reduce the DP IV or DP IV-analogous protein concentration in the mammalian organism. Effectors used according to the invention are, for example, DP IV inhibitors, such as the dipeptide derivatives and dipeptide mimetics alanyl-pyrolidide, isoleucyl-thiazolidide, and the pseudosubstrate N-valyl-prolyl, O-benzoyl hydroxylamine or salts thereof, especially fumarates thereof. Such compounds are known from the literature or can be manufactured analogously to the methods described in the literature (Demuth, 1990).

[0048] The method of the invention represents a novel procedure for raising lowered blood glucose concentration in the serum of mammals. It is simple, capable of commercial exploitation and suitable for use in human medicine in the treatment especially of diseases that result from below-average blood glucose values.

[0049] The effectors may be used in the form of pharmaceutical preparations that contain the active ingredient in combination with customary excipients known from the prior art and/or customary adjuvants. They are administered, for example, parenterally (for example *i.v.*, in physiological saline solution) or enterally (for example orally, formulated with customary excipients such as, for example, glucose).

[0050] Depending upon their endogenous stability and bio-availability and upon the severity of the condition, single or multiple doses of the effectors may be administered to obtain the desired normalisation of the blood glucose values. For example, in the case of aminoacyl-thiazolidides, such a dosage range may be from 0.1 mg to 10 mg of effector substance per kilogram. The effectors are preferably administered approximately 120 minutes after food intake. The effectors may also be used together with or at short intervals from glucagon or analogues thereof.

## Brief Description of Drawings

[0051] Figure 1 shows MALDI-TOF Mass Spectra of 0.14 mmol Glucagon Solution and 40 mmol TRIS/HCL (pH=7.6) in the presence of 40 nmol DP IV (the removal of the dipeptides His-Ser and Gln-Gly occurs sequentially depending upon the incubation time);

[0052] Figure 2 shows MALDI-TOF mass spectra of a glucagon-serum mixture; A-serum-



DP IV hydrolyses glucagon, B-suppression of DP IV catalyzed glucagon hydrolysis by the DP IV inhibitor, isoleucyl-thiazolidine;

[0053] Figure 3 shows the effect of glucagon on endogenous glucose release in Wistar rats after i.v. injection of glucagon, pre-incubated in plasma of DP IV-positive and DP IV-negative rats; and

[0054] Figure 4 shows the effect of glucagon on endogenous glucose release in Wistar rats after i.v. injection of glucagon, pre-incubated in plasma of normal rats, in the presence of absence of specific DP IV-inhibitor.

### Detailed Description

[0055] *Example 1: Inhibition of serum DP IV-catalysed glucagon cleavage by the DP IV inhibitor isoleucyl-thiazolidide*

[0056] See Figure 1

[0057] *Example 2: Effect of glucagon on endogenous glucose release after incubation in plasma of DP IV-positive and DP IV negative rats*

[0058] To test whether glucagon-degrading activity is present in the plasma of DP IV-negative rats, 6.8  $\mu$ g of glucagon were reinsulated for three hours at 37 °C in 1.0 ml of plasma of normal, DP IV-positive rats and in 1.0 ml of plasma of DPrats. From 10 to 50  $\mu$ l of the incubation solution were injected *i.v.* into normal Wistar rats and compared with a saline control. The biological response, that is to say the increase in blood glucose resulting from the glucagon-stimulated release of hepatic glucose, was monitored for 60 (Figure 3).

[0059] *Example 3: Effect of glucagon on glucose response in Wistar rats after i.v. injection of preincubated glucagon in the plasma of a normal rat, in the presence and absence of DP IV inhibitor*

[0060]

To test whether the effect of the glucagon-degrading activity in plasma can be inhibited by a specific DP IV inhibitor, 6.8  $\mu$ g of glucagon were incubated for three hours at 37 °C in 1.0 ml of normal rat plasma and in 1.0 ml of normal rat plasma additionally containing 0.01 mmol of isoleucyl-thiazolidide. From 10 to 50  $\mu$ l of the

[illegible]